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ISOLATION AND SEQUENCE ANALYSES OF SALINITY TOLERANCE GENES FROM BRUGUIERA CYLINDRICA (L.) BLUME

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By

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Faculty : Biotechnology and Biomolecular Sciences

Salinity is a major abiotic stress limiting the productivity of crop plants globally. The discovery of novel genes in stress adaptation will provide effective genetic engineering strategies leading to greater stress tolerance. The objectives of this research are to identify and isolate salinity tolerance genes from the mangrove plant, *Bruguiera cylindrica* (L.) Blume through suppression subtractive hybridization (SSH) and bacterial functional assay.

B. cylindrica propagules were grown in fresh water and 20 ppt salinity water. Root morphology differences between *B. cylindrica* grown in fresh water and 20 ppt salinity water were largely due to the need of roots to obtain more water and nutrients during salinity stress. *B. cylindrica* plants grew better in the presence of salt as higher mean values were obtained for all morphological measurements compared to *B. cylindrica* plants grown in fresh water.

Four RNA extraction methods were attempted to obtain high yield and high purity RNA. The cesium chloride method was chosen for RNA extraction as it gave the highest amount of pure RNA. Subtracted cDNAs were prepared from the roots of the *B. cylindrica* seedlings that were grown in fresh water and salt water, respectively. A total of 84 subtracted cDNAs were cloned into pCR-BLUNT II TOPO and sequenced. A total of 51 subtracted cDNAs with good sequencing quality were assembled into 7 contigs and 10 singletons. These non-redundant sequences were grouped into unknown protein (41.18%), novel (29.41%), protein destination and storage (11.76%), energy (5.88%), intracellular traffic (5.88%) and protein synthesis (5.88%). Some motifs of novel and unknown sequences may involve in the salinity tolerance of *B. cylindrica* such as Kv1.3 voltage-gated K^+ ion(s) channel signature, calcium-activated BK potassium channel alpha subunit and Kir2.1 inward rectifier K^+ ion(s) channel signature.

Meanwhile, a cDNA library was also constructed from the roots of *B. cylindrica* that were grown in fresh water. Bacterial functional assay was performed to identify cDNAs that confer salt tolerance. A total of 85 cDNA clones that were able to grow on 2× YT containing 400 mM NaCl were sequenced and 73 cDNAs with good sequence quality were assembled into 9 contigs and 53 singletons. The non-redundant sequences were also categorised into unknown protein (58.06%), metabolism (9.68%), transporters (9.68%), transcription (6.45%), energy (4.84%), cell growth/division (4.84%), novel (3.23%), miscellaneous (1.61%) and disease/defense (1.61%). A motif search on novel and unknown cDNA sequences had revealed some possible motifs that may be involved in salinity tolerance of *B. cylindrica* e.g. *C. elegans* Srg family integral membrane protein signature and 2Fe-2S ferredoxins, iron-sulfur binding region signature.

Sequence analysis of subtracted cDNAs and putative salt tolerant cDNAs isolated by bacterial functional assay showed some putative proteins that may be involved in the salinity tolerance of *B. cylindrica* such as putative potassium transporter HAK1p (M33), putative zinc finger protein (M3), ubiquitin (BC27) and L-ascorbate peroxidase (A46).

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMENCILAN DAN ANALISIS TURUTAN GEN-GEN TOLERANSI KEMASINAN DARI *BRUGUIERA CYLINDRICA* (L.) BLUME

Oleh

WONG YEEN YEE

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Kemasinan merupakan suatu tekanan abiotik utama yang menghadkan produktiviti tanaman di seluruh dunia. Penemuan gen-gen novel yang terlibat dalam adaptasi tekanan persekitaran akan menyediakan asas strategi pengubahsuaian genetik ke arah penyesuaian tanaman terhadap tekanan persekitaran yang lebih tinggi. Matlamat penyelidikan ini adalah untuk mengenalpasti dan memencilkan gen-gen dalam toleransi kemasinan dari pokok bakau, *Bruguiera cylindrica* (L.) Blume melalui strategi “suppression subtractive hybridization (SSH)” dan “bacterial functional assay”.

Biji benih vivipariti *B. cylindrica* disiram air biasa dan air bergaram sebanyak 20 ppt. Perbezaan morfologi akar yang terhasil di antara *B. cylindrica* yang disiram dengan air biasa dan air bergaram kemungkinan disebabkan oleh keperluan akar untuk memperolehi air dan nutrien yang lebih banyak semasa berada di dalam keadaan tekanan kemasinan. *B. cylindrica* membesar dengan lebih baik dengan kehadiran garam kerana nilai purata

ukuran morfologi yang lebih tinggi diperolehi dibandingkan dengan *B. cylindrica* yang disiram dengan air biasa.

Daripada empat kaedah pengekstrakan RNA yang telah dicuba, kaedah cesium klorida telah dipilih kerana kaedah ini memberi hasil RNA yang paling tulen dan tinggi. cDNA yang diekspres di dalam akar *B. cylindrica* yang membesar dengan kehadiran garam sahaja telah diperolehi melalui strategi SSH. Strategi SSH menyingkirkan cDNA di dalam akar *B. cylindrica* yang disiram dengan air biasa. Sejumlah 81 klon dari SSH telah diklon ke dalam pCR-BLUNT II TOPO dan dijujuk. Sebanyak 51 jujukan cDNA yang berkualiti dihimpunkan ke dalam 7 “contig” and 10 “singleton”. Jujukan-jujukan yang tidak redandensi ini dikumpulkan ke dalam kumpulan protin tidak diketahui (41.18%), unik (29.41); destinasi and penyimpanan protin (11.76%), tenaga (5.88%), trafik intrasel (5.88%) dan protin sintesis (5.88%). Motif yang dimiliki oleh jujukan unik dan tidak diketahui mungkin terlibat di dalam toleransi kemasinan pokok bakau *B. cylindrica* seperti Kv1.3 voltage-gated K^+ ion(s) channel signature, calcium-activated BK potassium channel alpha subunit and Kir2.1 inward rectifier K^+ ion(s) channel signature.

Selain itu, satu perpustakaan cDNA telah disediakan daripada akar *B. cylindrica* yang disiram dengan air biasa. “Bacterial functional assay” telah digunakan untuk mengenalpasti cDNA yang mempunyai sifat toleransi kemasinan. Sejumlah 85 klon yang tumbuh di atas plat 2× YT telah dipencil dan dijujuk. Sebanyak 73 jujukan yang berkualiti dihimpunkan ke dalam 9 “contig” and 53 “singleton”. Jujukan-jujukan yang tidak redandensi turut dikategorikan ke dalam kumpulan protin tidak diketahui (58.06%), metabolime (9.68%), pengangkutan (9.68%), transkripsi (6.45%), tenaga (4.84%), pertumbuhan and pembahagian sel (4.84%), unik (3.23%), lain-lain (1.61%) serta penyakit dan pertahanan (1.61%). Carian motif ke atas jujukan unik dan tidak diketahui telah memberi gambaran terhadap motif yang mungkin terlibat di dalam toleransi kemasinan *B. cylindrica* seperti *C. elegans* Srg family integral membrane protein signature and 2Fe-2S ferredoxins, iron-sulfur binding region signature.

Analisa penjujukan cDNA dari SSH dan “bacterial functional assay” menunjukkan kehadiran protin-protin yang mungkin terlibat di dalam toleransi kemasinan pokok bakau, *B. cylindrica* seperti “putative potassium transporter HAK1p” (M33), “putative zinc finger protein” (M3), “ubiquitin” (BC27) and “L-ascorbate peroxidase” (A46).

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I certify that an Examination Committee met on 25th July 2005 to conduct the final examination of Wong Yeen Yee on her Master of Science thesis entitled "Isolation and Sequence Analyses of Salinity Tolerance Genes from *Brugeiera cylindrical* (L.) Blume" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science degree. The members of the Supervisory Committee are as follows:

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DECRALATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

WONG YEEN YEE

Date: 19/10/05

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LIST OF ABBREVIATIONS

β	beta
λ	lambda
×*g*	gravitational acceleration
μg	microgram
μL	microliter
°C	degree centigrade
%	percentage
AMV	avian myeloblastosis virus
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
Ca	calcium
cDNA	complementary DNA
CIP	calf intestinal phosphatase
Cl	chloride
cm	centimeter
CsCl	cesium cholride
CTAB	hexacetyltrimethyl ammonium bromide
dATP	2'-deoxy-adenosine-5'-triphosphate
dCTP	2'-deoxy-cytidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
dGTP	2'-deoxy-guanosine-5'-triphosphate
DNA	deoxyribonucleic acid

DNase	deoxyribonuclease
dNTPs	deoxynucleotides
ds	double-stranded
DTT	dithiothreitol
dTTP	thymidine-5'-tryphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
g	gram
HCl	hydrochloric acid
K	potassium
kb	kilo base pairs
L	liter
LB	Luria-bertani
LiCl	lithium chloride
M	molar
Mg	magnesium
$MgSO_4$	magnesium sulfate
mL	milliliter
mM	millimolar
mRNA	messenger RNA
Na	sodium
NaCl	sodium chloride
NaOAc	sodium acetate
NaOH	sodium hydroxide
NCBI	National Center for Biotechnology Information

ng	nanogram
NH_4Oac	ammonium acetate
OD	optical density
PCR	polymerase chain reactions
pfu	plaque forming units
ppt	part per thousand
RNA	ribonucleic acid
RNase A	ribonuclease A
ROS	reactive oxygen species
rpm	revolution per minute
SC-U	SC minimal medium without uracil
SDS	sodium dodecyl sulphate
SOS	salt overly sensitive
SSH	suppression subtractive hybridization
U	units
v/v	volume per volume
w/v	weight per volume
YPD	yeast extract peptone dextrose medium
YT	Yeast extract tryptone medium

CHAPTER 1

INTRODUCTION

Salinity is one of the major abiotic stress limiting plant productivity and growth globally. Salinity imposes osmotic stress and ionic stress to the plants. Irrigation practices in agricultural lands have steadily increased the concentration of salt in the soil (Khan and Duke, 2001). Unfavorable physiochemical environments can cause average losses more than 65% of optimal yields (Boyer, 1982). More efficient and productive agriculture will be possible on salt affected soils if crop plants with improved salinity tolerance can be selected and bred through traditional breeding or genetic engineering.

As halophytes can live under high salinity condition, it is advantageous to identify genes that are involved in the salinity tolerance of these plants to adapt to harsh environment. Mangroves are unique communities along the tropical and sub-tropical coastal regions that are formed by almost fifty unrelated plant species (Banzai *et al*., 2002a). Mangroves are divided into two groups based on their morphological features of salt management i.e. 'secreters' and 'non-secreters' (Tomlinson, 1986). The 'secreters' possess salt glands or salt hairs to eliminate excess salt from plants while the 'non-secreters' have no such morphological devices (Tomlinson, 1986; Banzai *et al*., 2002a).

In this study, *Bruguiera cylindrica* (L.) Blume, known locally in Malaysia as "bakau putih", was chosen as a source to study novel salinity tolerance genes in halophytes. *B. cylindrica* is categorized as one of the 'non-secreters' and it tolerates high salinity through the ultrafiltration system whereby the plant is able to exclude a large proportion of salt from the water it uptakes and selectively absorbs only certain ions (Tomlinson, 1986).

The objectives of this study were to identify genes that are involved in salinity tolerance of *B. cylindrica* using suppression subtractive hybridization (SSH) and bacterial functional assay. By using the SSH method, genes that were expressed only in the root of *B. cylindrica* grown in the presence of NaCl can be identified. Meanwhile, *B. cylindrica* cDNAs that confer salt tolerance to bacteria can be isolated by performing bacterial functional assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Stress in plants

Stress results changes in plant physiology caused by one or more environmental and biological factors (Hale and Orcutt, 1987). Table 1 shows the sources of environmental stress for plants (Hale and Orcutt, 1987). Stresses are divided into two groups i.e. abiotic and biotic stress. Abiotic stress depends on geographical and climatic differentiation such as annual rainfall differences, chilling, heat, drought, salinity, flooding and freezing (Holmberg and Bülow, 1998; Abeysinghe *et al*., 2000). Whereas, examples of biotic stresses are diseases and pests.

2.1.1 Salt and drought stress

Salinity and drought are the two major environmental factors that reduce plant productivity currently (Serrano *et al*., 1999). Boyer (1982) mentioned that disease and insect damages cause losses of less than 10% while unfavorable physiochemical environments cause losses of more than 65%. From historical records, civilizations had never progressed in one locality for more than 1000-2000 years because of the destruction of the resource base of the area (Ashraf, 1994). As a result of poor water management, civilizations have been destroyed by the accumulation of salt on the soils. If there is limited rainfall, salt is not leached out of the soil. Crop plants take in salt through their roots and yields are reduced as the salt concentration increases. Drought and salinity are interconnected because crop production

Table 1. Sources of environmental stress for plants (reproduced from Hale and Orcutt, 1987).

Physical	Chemical	Biotic
Drought	Air pollution	Competition
Temperature	Allelochemicals	Allelopathy
Radiation	(organic)	Lack of symbioses
Flooding	Nutrients (inorganic)	Human activities
Mechanical	Pesticides	Diseases
Electrical	Toxins	Insects
Magnetic	Salts	
Wind	pH of soil solution	